Microbiota of the Planalto de Bolona: an artisanal cheese produced in uncommon environmental conditions in the Capo Verde islands

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Microbiota of the *Planalto de Bolona*: an Artisanal Cheese Produced in Uncommon Environmental Conditions in the Cape Verde Islands

Valentina Alessandria[1], Paola Dolci[1], Kalliopi Rantsiou[1], Daniele Pattono[2], Alessandra Dalmasso[2], Tiziana Civera[2] and Luca Cocolin[1*]

[1]Di.Va.P.R.A., Faculty of Agriculture, University of Turin, Italy

[2]Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin, Italy

*Corresponding author: Di.Va.P.R.A., via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy. Phone: 0039 011 670-8553, fax 0039 011 670-8549, email: lucasimone.cocolin@unito.it

Abstract

The present study aimed to evaluate the dominant microbial community naturally present in the *Planalto de Bolona* cheese, produced in the Cape Verde Islands. Samples of milk, curd and cheese from two different producers were examined through culture-dependent and independent-methods. Traditional plating and genetic identification of lactic acid bacteria (LAB) and yeast isolates were carried out. Moreover, DNA and RNA extracted directly from samples were subjected to Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE).

Concerning the LAB population, a total of 278 isolates were identified: *Lactococcus lactis* subsp. *lactis* and *Enterococcus faecium* represented the most isolated species. Regarding yeasts, the analysis of isolates throughout the manufacturing period showed a consistent presence of the genus *Candida*. Divergences in species detection between culture-dependent and culture-independent methods were observed, as well as between DNA and RNA analysis. PCR-DGGE underlined high heterogeneity among bacterial species while yeast microbiota was dominated by *Aureobasidium pullulans* at DNA level. The obtained results represent a first approach in the understanding of the *Planalto de Bolona* cheese microbial ecology and consequently may constitute a first step towards the full comprehension of the microbiota of this artisanal cheese produced in unusual environmental conditions in the Cape Verde Islands.

Keywords: *Planalto de Bolona*, ecology, PCR, RT-PCR, DGGE, 16S rRNA sequencing

Introduction

The *Bolona cheese* is produced in the Cape Verde Islands, specifically in the Planalto de Bolona, a desert mountain area in the Island of Sant'Antao, where goat breeding represents one of the main sources of employment for the local population. The Bolona cheese is a pressed, fresh cheese produced from goat-milk, coagulated by addition of home made kid's rennet, usually without any ripening. Its manufacturing is highly influenced by the lack of water resources characterising the desert environment in which it is produced. As water is scarce, the washing of cheese-making equipment is usually performed with whey. Cheese-making starts immediately after milking in tiny traditional stone huts with roofs of straw and matting. The milk transformation is often an open-air process, obtained without any thermal treatment or starter culture addition. Once the curd is ready, it is broken down to the size of corn grains and then separated from the whey, pressed by hand and salted. The characteristic shape of the Bolona cheese is obtained using a tuna-like can, and weighs about 400 g. Salted using local sea-salt, the cheese is usually sold and consumed within 72 hours.

The microbial population naturally present in cheeses constitutes an important parameter involved in the definition of the composition, structural and sensorial characteristics of a specific cheese. The microbiota of each dairy product (as well as each fermented food) has its own history, during which the microbial population structure changes under the influence of continuous shifts in environmental factors occurring during its preparation (Coppola et al 2008). The microbiota of cheeses is mainly represented by lactic acid bacteria (LAB), which have important roles in their manufacturing. In seasoned cheeses, LAB have an impact both during the fermentation process, as well as during the later steps of the production, especially during seasoning, in terms of aroma production. In fresh cheeses, LAB populations are responsible for the acidification process, thereby preventing the growth of spoilage microorganisms, and for the development of aroma profiles associated with buttery and cream notes (Fox et al 2004). Following the above assumptions, the aim of this research was to investigate the indigenous bacterial population involved in the production of the Bolona cheese, in order to obtain preliminary knowledge of lactic acid bacteria (LAB) species

carrying out its fermentation in the peculiar environmental conditions described. The work also monitored the population of yeasts, which plays a significant role through their ability to metabolize milk constituents (Fleet 1990). The study adopted both culture-dependent and independent methods, in order to obtain a wider knowledge on the Cape Verde Bolona cheese microflora. LAB and yeasts, isolated by conventional methods, were identified by molecular methods. At the same time, total DNA and RNA were directly extracted from samples and Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was carried out to understand the composition of dominant microbiota in food matrices. Analysis of RNA through reverse transcription PCR (RT-PCR) and DGGE was carried out to reveal and monitor the metabolically active microorganisms.

Material and methods

Dairy samples and microbiological analysis

Samples of milk (M), curd (Cu) and cheese (Ch) produced in the Cape Verde Islands were collected from two different productions, each from two different producers. In this study the two producers are called P1 and P2 and, for each of them, A and B represent the two productions. A total of 6 samples of milk, curd and cheese, 3 for each production, and for each producer, were included in the study and analyzed. Samples were picked up in the winter season with a difference of about 2 months from each sampling. All samples were transported to the laboratory in Italy under strict refrigeration conditions and subjected to microbiological analysis. Samples were analyzed in by conventional microbiological methods. Ten millilitres of milk and ten grams of curd and cheese samples were homogenized in 40 ml sterile Ringer solution (Oxoid, Milan, Italy) with a stomacher machine, serially diluted in Ringer solution and plated on specific media for viable counts. The following analysis were carried out on duplicate agar plates: i) total aerobic mesophilic flora on Gelatin Peptone Agar (GPA) (Oxoid), incubated for 48 h at 30 °C; ii) mesophilic and thermophilic cocci on M17 (Oxoid), incubated respectively at 22 and 42°C for 48 h; iii) mesophilic (22°C) and

thermophilic lactobacilli (42°C) on de Man Rogosa Sharpe (MRS) agar (Oxoid); iv) enterococci on Kanamycin Aesculin Azide Agar (KAA) (Oxoid), at 37°C for 24 h; v) coagulase-negative cocci (CNC) on Mannitol Salt Agar (MSA) (Oxoid), at 30°C for 48 h; vi) total coliforms on Violet Red Bile Lactose Agar (VRBLA) (Fluka), at 37°C for 24 h; fecal coliforms on VRBLA at 42°C for 24 h; vii) yeasts on Malt Agar (Oxoid) supplemented with tetracycline (1 µg/ml, Sigma, Milan Italy), at 25°C for 96 h. After counting, means and standard deviations were calculated.

A total of 25 randomly selected colonies from each M17 agar, MRS agar and KAA agar, as well as 10 colonies from Malt Agar, were isolated for each type of sample, in order to obtain a representative LAB and yeast population of Cape Verde Cheese. They were purified respectively on M17 agar, MRS agar, KAA agar and Malt agar and stored at -80°C in M17 broth for lactococci and enterococci, in MRS for lactobacilli and in YPD (1% yeast extract, 2% peptone and 2% glucose) for yeasts.

DNA extraction from pure cultures

Genomic DNA was extracted from 1 millilitre of an overnight culture of each isolate and centrifuged at 13,200 rpm for 10 min at 4°C to pellet the cells. The pellet was subjected to DNA extraction according to Rantsiou et al (2008). In the case of LAB, a treatment of 30 min at 37°C with lysozyme (50 mg/ml, Sigma) was carried out for cell lysis.

Direct extraction of nucleic acids from dairy samples

For curd and cheese samples, 10 g were homogenized in a stomacher bag with 40 ml of Ringer solution. Big debris was allowed to deposit for 5 min and 1 ml, for both RNA and DNA, of supernatant was collected and centrifuged at 13,200 rpm for 10 min to pellet the cells. For milk, 1 ml, for both RNA and DNA, was directly centrifuges and the pellet used for nucleic acids extraction.

After centrifugation, the pellet was re-suspended in 150 µL of proteinase K buffer (50 mM Tris-

HCl, 10 mM EDTA pH 7.5, 0.5% [wt/vol] sodium dodecyl sulphate) and 25 µL of proteinase K (25 mg/ml, Sigma) and 50 µL of lysozyme (50 mg/ml, Sigma) were added. The solution was transferred to 1.5 ml screw cap tube, containing 0.3 g of glass beads with a diameter of 0.5 mm, and submitted to heat treatment at 50°C for 1 h. Later on, 150 µL of 2X breaking buffer (4% [vol/vol] Triton X-100, 2% [wt/vol] sodium dodecyl sulphate, 200 mM NaCl, 20 mM Tris pH 8 and 2 mM EDTA pH 8) as well as 300 µL of phenol-chloroform-isoamyl alcohol 25:24:1 (pH 6.7, Sigma) in the case of DNA, or 300 µL of phenol-chloroform 5:1 (pH 4.7, Sigma) for RNA, were added and the tubes were submitted to three 30-s bead beater (Fast Prep; Bio 101, Ca, USA) treatments at 4.5 m/sec speed. Three hundred microlitres of TE (10 mM Tris, 1mM EDTA) were added in the tubes and a centrifugation at 13,200 rpm for 10 min was performed. The aqueous phase was collected and nucleic acids were precipitated by the addition of 750 µL ice-cold absolute ethanol. DNA and RNA were pelleted by centrifugation at 13,200 rpm for 10 min at 4°C, washed briefly in 70% ethanol and re-suspended in 50 µL of sterile water. In the case of RNA, DNase-free RNase (Ambion, Monza, Italy) was added and the RNA samples were incubated at 37°C for 3 h to digest DNA. RNA samples were checked for the presence of residual DNA by PCR amplification (Cocolin et al. 2001 b).

PCR and RT-PCR

DNA extracted from bacteria isolates was amplified using PCR with a primer set designed by Klijn et al. (1991): P1V1 (5'- GCG GCG TGC CTA ATA CAT GC-3') and P2V1 (5'TTC CCC ACG CGT TAC TCA CC-3'), while yeast DNA was amplified with primers NL1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and LS2 (5'- ATT CCC AAA CAA CTC GAC TC-3'), as reported by Cocolin et al. (2000). A GC-clamp (5'- CGC CCG CCG CGC GCG GCG GGC GCG GGG GCA CGG GGG G-3') was attached to the P1V1 and NL1 primers for DGGE analysis, as described by Sheffield et al. (1989). Both PCR reactions were performed in a final volume of 25 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside

triphospates (dNTP), 1.25 U of Taq Polymerase (Applied Biosystems, Milan Italy), 0.2 µM of each primer and 100 ng of template DNA. Amplifications were carried out in a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy) as described by Cocolin et al. (2001b) for bacteria and Cocolin et al. (2000) for yeasts.

To investigate the samples for the dominant bacterial species, the variable V3 region of 16S rRNA gene was amplified with primers 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5'- ATT ACC GCG GCT GCT GG-3') (Ampe et al. 1999). A GC clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the end of 5' of primer 338f for DGGE analysis. The PCR was performed in a final 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphospates (dNTP), 1.25 U of Taq Polymerase (Applied Biosystems, Milan Italy), 0.2 µM of each primer and 100 ng of DNA. The amplification cycle of denaturation at 95°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min was repeated 35 times. The cycle was preceded by an initial denaturation at 95°C for 5 min and followed by a final extension at 72°C for 10 min.

Concerning yeasts DNA, primers NL1 with GC clamp and LS2 were used. The reaction mix was the same used for yeast isolates as described above, adding 100 ng of DNA.

The reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). One microgram of RNA was mixed with 1 µL of 10 µM of primer 518r or LS2, for bacteria and yeast RNA, respectively, and sterile water to a final volume of 10 µL and incubated at 75°C for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris- HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM of each dNTP, 1 µL of 200 U/µL M-MLV and 0.96 units of RNasin ribonuclease inhibitor (Ambion) was transferred to the reaction tube. The reverse transcription was carried out at 42°C for 1 h and 1 µL of RT reaction was added to the PCR reaction mix.

DGGE analysis

The Dcode universal mutation detection system (Biorad, Milan, Italy) was used for DGGE analysis. PCR products of bacteria and yeast isolates were analyzed by DGGE as described by Cocolin et al. (2001b) and by Cocolin et al. (2000), respectively.

PCR products obtained with primers 338f/518r were applied to a 8% (wt/vol) polyacrilamide gel (acrylamide-bis acrylamide 37:5:1) with a denaturing gradient from 30 to 60% (Cocolin et al. 2001a), while in the case of PCR products obtained with primers NL1/LS2 the gradient was from 30 to 50% in a 1X TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8]). Gels were subjected to a voltage of 130 volt for 4h at 60°C, stained for 20 min in 1x TAE containing 1x SYBR Green I (Sigma) and then analyzed under UV by using UVI pro platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany).

Molecular identification of the isolates

DGGE profiles of isolates were grouped and representatives of each group were amplified with primers P1V1 and P4V3 (Klijn et al. 1991) targeting the 700 bp of the V1-V3 region of the 16S rRNA gene for bacteria DNA and with primers NL1/ NL4 (Kurtzman and Robnett 1997) for yeast DNA to amplify partial 26S rRNA gene. The PCR products were sent to MWG Biotech (Edersberg, Germany) for sequencing and the resultant sequences were aligned with those in Gene Bank using the Blast program (Altschul et al. 1997), to determine the known relatives.

Sequencing of bands

The program BioNumerics Software (Applied-Maths, Sint-Martens-Latem, Belgium) was used for pattern analysis and for normalization of gels. Selected DGGE bands were extracted from the gels using sterile pipette tips, transferred into 50 µL sterile water and incubated overnight at 4°C. Two µL of the eluted DNA were re-amplified by using the conditions described above and checked by DGGE. PCR products that gave a single band, comigrating with the DNA/RNA control, were then amplified with bacteria and yeast primers without GC clamp and sequenced by a commercial

facility (MWG Biotech, Ebersberg, Germany). Sequences were aligned in Gene Bank using the Blast Program (Altschul et al. 1997). For those bands whose sequencing did not produce any result, cloning procedure was performed. PCR products were cloned in pGEM-T Easy vector (Promega, Milan, Italy) and the resulting colonies were checked also in this case by DGGE using previously amplified DNA and RNA extracted directly from the samples as a control. Only clones migrating as a single band and at the same position with respect to the control were sequenced at MWG Biotech and sequences were aligned in Gene Bank for identification purposes.

Results

Microbial dynamics as obtained by plate counts

The mean microbial counts and standard deviation of dairy samples of Cape Verde are reported in Table 1. Traditional microbiological analysis showed increasing counts from the milk to the cheese in the productions of samples of both producers (P1-P2). The mesophilic aerobic bacteria count ranged from 3 log cfu/ml in milk and reached the maximum value of 6.25 cfu/g in the cheese of the second producer.

Concerning the counts of presumptive lactococci and lactobacilli on M17 and MRS agar plates, similar trends were observed. Furthermore no differences were observed between mesophilic and thermophilic LAB. Differences were detected in the counts of the two producers: while in the milk the values of both P1 and P2 were similar ranging from 2.4 to 3.8 log cfu/ml, in the curd and in the cheese the counts of P2 presented higher values than those of P1 in all media. The highest value (7.5 log cfu/g) was reached in the cheese in M17 at 22°C.

As far as the enterococci are concerned, the counts were lower than those of lactococci and lactobacilli (maximum value: 4.3 log cfu/g).

The CNC reached the highest level in the cheese of both producers, with values of about 4.7 log cfu/g, while yeasts reached counts of 3.90 log cfu/g in cheese of P2. Total and fecal coliforms were present with similar counts and they presented higher values in the products of P2 (4.29 log cfu/g in

cheese).

Molecular identification of LAB and yeast isolates

The number and percentage of LAB and yeast isolates among samplings are reported in Tables 2 and 3.

Concerning LAB population, a total of 278 isolates were randomly selected from agar plates and subjected to molecular identification. *Lactococcus lactis* subsp. *lactis* (27.6% of the LAB population in P1 and 43% in P2) and *Enterococcus faecium* (39.4% in P1 and 35.1% in P2) represented the most isolated species. In addition, both productions showed a high heterogeneity of minor species, counting for the 33% in P1 and 22% in P2. For what concerns lactobacilli, *Lactobacillus pentosus* showed the highest percentages in both cases (9.4% in P1 and 6.6% in P2), while *Lactobacillus plantarum* and *Lactobacillus brevis* were found only in samples of P1 and with lower values (respectively 1.6% and 3.1%). Other enterococci species were isolated only in P2 and, in particular, in samples of milk: *Enterococcus casseliflavus* presented the highest occurrence (9.3% of the total LAB in P2), while *Enterococcus italicus* and *Enterococcus durans* were encountered in lower values. *Enterococcus faecalis* was isolated from curd and cheese. Other relevant values were highlighted for *Leuconostoc mesenteroides* (9.4% in P1).

In the P1, *L. lactis* subsp. *lactis* and *E. faecium* showed their highest values in the milk and then slightly decreased in the curd and in the cheese. In particular, the milk of P1 was characterised by a low presence of minor species (*Leuc. mesenteroides, Weisella paramesenteroides, Leuconostoc citreum* and *Streptococcus parauberis*), progressively increasing through the manufacturing in the curd and cheese, together with the development of other species (*Lb. pentosus* as the most noticeable). *S. parauberis* and *Leuc. citreum* were isolated only in the milk. On the other hand, the milk of P2 presented instead a higher balance in the shares of the different species, with the minor species strongly diminishing throughout the manufacturing (from 51.4 % in the milk to 12.7 % in the cheese). In this case, *L. lactis* subsp. *lactis* and *E. faecium* presented lower shares in the milk

(also due to the high presence of *E. casseliflavus*), then raising in the curd and in the cheese.

Regarding yeasts, a total of 69 isolates were identified. The analysis of isolates throughout the manufacturing showed a consistent presence of the genus *Candida* in the samples of P1 (*Candida pararugosa* with 41.5%, *Candida zeylanoides* with 24.4% and *Candida parapsilosis* with 17.1%). The remaining share was constituted by *Trichosporon coremiiforme* present in milk, curd and cheese with the same percentage and *Debaryomyces hansenii* (isolated only in the milk). In the samples of P2, a high presence of minor species (*Aureobasidium pullulans, C. pararugosa*, *Cryptococcus diffluens* as the most noticeable) was observed in the milk. The *Rhodotorula glutinis* was found consistently in the milk then decreasing in the curd and in the cheese due to the development of *C. parapsilosis*.

DGGE analysis of samples

Total DNA and RNA from all samples were extracted and analysed by PCR-DGGE. The DNA and RNA gels are shown in Figures 1, 2, 3 and 4 while the results of the identification of bands are presented in Tables 4 and 5.

For what concerns the bacteria population, a high variability was observed in the samples of the two producers. Bands corresponding to *L. lactis* subsp. *lactis* (4-9-12-20-21-24-27, Figs.1 and 2) were the most frequent in the samples of both P1 and P2, in DNA as well as in RNA. Regarding the other species, a high heterogeneity was observed (Figs. 1 and 2): *Lactobacillus helveticus* (bands 13 and 14) was only found in P1 curd samples at RNA level, and several other microorganisms appeared only once. This was noticed for *Leuconostoc pseudomesenteroides* (band 16), *Moraxella osloensis* (band 11) and *Kocuria rhizophila* (band 17), found at RNA level, and *Propionibacterium acnes* (band 7), *Gluconobacter thailandicus* (band 3) and *Methylobacterium* sp. (band 2), found for the DNA. *Escherichia coli* (band 6 and 22, Figs. 1 and 2) was only identified at DNA level in one production of both P1 and P2, and only in samples of cheese. Although P2 presented a lower number of different species, *Klebsiella* sp. (band 23) and a *Firmicutes* population (band 26) were

found only in P2 samples.

Primers used for bacterial DNA analysis amplified eukaryotic DNA as well, highlighting the presence of *Saccharomyces cerevisiae* (only in P1), *Sus scrofa* mRNA and *Delphinella strobiligena* (in both P1 and P2).

Regarding yeast/mould population at DNA level, all the samples showed three bands that were impossible to separate. After cloning and sequencing, the band 1 (Fig. 3) resulted to be *A. pullulans*. Other bands were cut and resulted as *Phoma herbarum* (band 2, Fig. 3), *Seyrigia humbertii* revealed in a sample of curd (band 3, Fig. 3) and *S. cerevisiae* revealed in cheese (band 6, Fig. 4).

In the case of RNA, a higher variability emerged. Bands 11 and 12 (Figs. 3 and 4) were identified as *Rhizomucor miehei*, and were found in samples of curd and milk. *S. cerevisiae* (band 7, 8, 15, 4 and 5) was the most present species found at RNA level as shown in Figs. 3 and 4 and it was present in samples of both producers in milk, as well as in curd and cheese. *Filobasidium elegans* (band 9, Fig. 4) was found only in production B of P2 while *Alternaria alternata* was found in the cheese A of P1 (band 14, Fig. 3) and once in the curd of P2 (band 13, Fig.4). *C. pararugosa* was observed in cheese B of P1 (band 10) and in the milk B of P2 (band 16).

Discussion

This study provides a first overall analysis of the microbial communities in the milk and of their evolution in the curd and in the cheese produced in Cape Verde Islands.

The conventional microbiological data highlighted a consistent count variation also between the productions of each producer, as suggested by the high standard deviation of the average counts. This may be due to the differences in terms of milk as well as to the low standardization of the peculiar technology of production. *L. lactis* subsp. *lactis* and *E. faecium* resulted the most frequent species on agar plates, with *L. lactis* subsp. *lactis* representing one of the most predominant species in cheese-making, as it is the case for many European artisanal dairy products (Cogan et al.1997). Concerning enterococci (*E. faecium*, together with *E. italicus*, *E. durans*, *E. faecalis* and *E.*

casselliflavus present in lower shares), their presence is widely reported in artisanal dairy products (Cogan et al. 1997; Dolci et al. 2008a 2008b). At the same time, their presence could be also associated to the low hygienic conditions during milking and storing processes (Garcia Fontan et al. 2001), and in this case their occurrence may also be explained by the peculiar environmental conditions and the adopted production technology. Counts on MRS plates, a medium used to enumerate lactobacilli, resulted similar to those on M17, but the molecular identification showed that only a minimal share was represented by lactobacilli. This may be due to the low specificity of MRS medium, where also lactococci species grew. Supporting this hypothesis, it is interesting to point out how several authors already highlighted the scarce selectivity of the used media (Ampe et al. 1999; Randazzo et al. 2002; Ercolini et al. 2003; Rantsious et al. 2008). Moreover, on these selective media also species of staphylococci and *W. paramesenteroides* grew.

Also in the case the of the culture independent method, species of lactobacilli represented a minority. They were found only at RNA level and only in the curds sample of P2. The scarce presence of lactobacilli is explainable considering that the Bolona is a fresh cheese and lactobacilli occurrence is usually higher in ripened semi-hard cheeses (Beresford et al. 2001). Culture-independent methods were also used to overcome the problems usually associated with microflora growth on media (Ercolini 2004). This approach did not prove to be relevant in the case of *L. lactis* subsp. *lactis,* which was the most recurrent species throughout the manufacturing, as we observed from results obtained by plating. On the other hand for the majority of the other species, relevant differences were observed. For instance, enterococci present on the plates did not produce any bands in DGGE gels, most probably due to the detection limit of the PCR-DGGE method, which has been reported to be 10^3-10^4 cfu/g or ml (Cocolin et al 2001b). Moreover, several species not present on the plates were identified through the culture-independent method, and in particular *Methylobacterium* sp., *G. thailandicus, K. rhizophila, M. osloensis, P. acnes, E. coli, Klebsiella* sp. Some of them are usually present in cheeses, while others are very unusual in food and in particular, in cheeses.

The primers 338f-518r targeting the bacterial 16S rRNA gene V3 variable region did not present a high specificity and amplified eukaryotic species as well, in this way identifying *S. cerevisiae, D. strobiligena* and *Sus scrofa* mRNA. This phenomenon has been already highlighted in a study by Lopez et al. (2003), where it was shown that primers for the V3 region could also amplify yeast and fungal species. The presence of *Sus scrofa* mRNA in goat cheese is very odd, and may be once again explained if we consider the specific environmental and technological conditions characterizing the production of the Bolona cheese, in which animals of different species are sharing the same farm spaces.

Literature analysis shows that few contributions concerning population dynamics took in consideration DGGE fingerprint obtained from RNA (Randazzo et al. 2002; Rantsiou et al. 2008). In this study, RNA was analyzed to monitor the active microflora and evaluate differences from DNA, which is usually used in order to profile the biodiversity within a specific ecosystem. Only a partial correspondence was revealed: *L. lactis* subsp. *lactis* was found also in RNA underlining its vitality. *M. osloensis*, *Lb. helveticus*, *Leuc. pseudomesenteroides*, and *K. rhizophila* and *Firmicutes* population were found only at RNA level.

Regarding yeasts, the analyses showed maximum values of 4 log cfu/g, confirming yeasts' ability to multiply in dairy products, despite their low values. Yeasts are usually detected in dairy products reflecting a good adaptation to a substrate rich on proteins, lipids, sugars and organic acids. In addition, yeasts are able to grow in substrates with high salt concentration, low temperatures, low pH and water activity (Lopandic et al. 2006). The occurrence of yeasts in cheeses may contribute positively to flavour development during the stage of maturation or, on the contrary, may lead to product spoilage (Fleet 1990; Pereira 2000 et al. 2000; Corbo et al. 2001). However, in this case the short shelf-life of the Bolona cheese induce to presuppose a scarce yeasts contribution to the characteristic of the product.

The identification of yeast isolates showed a high number of genus *Candida*. In particular, in samples of P2, a high percentage was represented by *C. parapsilosis*. This result is in apparent

contradiction with their low ability to survive in cheeses (Jacques and Casaregola 2008).

A completely different picture regarding the yeast ecology was obtained with the culture-independent approach. Apart for *C. pararugosa*, that was detected in one RNA cheese sample, no other *Candida* species were observed by DGGE. Furthermore, differences were observed between DNA and RNA. In particular DNA was characterized by the presence of *A. pullulans* in all samples (while in plates the latter was found only in milk of P2), this being attributable to specific environmental contaminations. On the other hand, the main yeast species observed at RNA level was *S. cerevisiae*, which was present in samples of both producers. This is not unusual, as the presence of *S. cerevisiae* has been reported in several fermented dairy products and cheeses (Bankole and Okagbue 1992; Roostita and Fleet 1996; Prillinger et al. 1999; Gadaga et al. 2000; Abdelgadir et al. 2001; Jespenser 2003). Overall, the DGGE profiles at both DNA and RNA level for the two producers were rather similar.

Divergences microbial species detection between culture-dependent and culture-independent methods could be due to different reasons: for example, the permanence in cheese matrix of DNA coming from cellular autolysis, or the high selectivity of some media towards specific microorganisms that find optimal conditions for their growth (Dolci et al. 2008a). The differences highlighted between the results obtained through the different methods confirmed the importance to combine molecular culture-independent methods with classical microbiological analysis in the study of complex microbial communities of the food matrices as reported in many other studies (Ercolini 2004; Dolci et al. 2008a; Rantsiou and Cocolin 2006; Rantsiou et al. 2008). In conclusion, the importance for the present research lies also in the fact that, within the African environmental contexts, fermented milk products are of great significance in their therapeutic value for alleviating lactose intolerance, their social value and as a means of generating income (Beukes et al. 2001). In this concern, the obtained results constitute the first attempt to provide an overview of the indigenous microbiota of the Bolona cheese. They may constitute an important set of information to contribute to the improvement of the cheese-making production conditions in the characteristic

environment of Cape Verde Islands, by providing autochthonous strains which can be used as starter cultures to increase the standardization and quality of this artisanal product.

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Table 1. Microbial counts (expressed as mean of log cfu/mL for milk and log cfu/g for curd and cheese of the 2 productions) and standard deviations (SD) of samples of P1 and P2. Refer to materials and methods for media specifications and incubation conditions.

	GPA		M17 22°C		M17 42°C		MRS 22°C		MRS 42°C		KAA		MSA		MALT AGAR		VRBLA 37°C		VRBLA 42°C	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
P1-M	3,36	0,31	3,51	0,33	3,23	0,21	3,17	0,14	2,46	0,31	1,21	0,24	3,04	0,21	2,15	0,11	1,55	0,21	0,57	0,81
P1-Cu	4,54	0,50	4,47	0,15	4,31	0,07	4,48	0,30	4,10	0,59	1,06	0,40	3,91	0,80	2,68	0,99	< 5[a]	n.a[b]	<5 [a]	n.a[b]
P1-Ch	5,59	2,01	5,70	0,60	4,84	0,67	5,45	0,62	4,45	0,49	1,69	0,77	4,64	1,40	3,15	1,53	0,53	0,92	0,65	0,92
P2-M	3,49	2,33	3,79	2,16	3,41	2,14	2,65	2,40	2,52	1,36	1,63	2,30	1,77	1,1	2,00	0,3	1,48	0,74	1,40	1,23
P2-Cu	4,30	0,91	5,30	0,99	5,00	0,87	5,09	1,29	4,51	2,32	4,36	2,06	3,58	1,6	3,05	3,1	3,25	2,26	3,05	2,90
P2-Ch	6,25	1,63	7,53	2,00	6,76	2,11	7,01	2,87	5,01	1,82	3,88	0,70	4,77	2,6	3,90	4,8	4,29	3,89	4,25	3,83

[a]Count expressed in cfu/g

[b]n.a., “not applicable”

Table 2. Molecular identification of LAB isolates of P1 and P2. The isolates were selected randomly, grouped based on co-migration in PCR-DGGE and representatives of each group were sequenced to obtain an identification for the whole group.

LAB isolates	**M1**		**Cu 1**		**Ch 1**		**%Total P1**		**M2**		**Cu 2**		**Ch 2**		**% Total P2**	
	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%
Enterococcus faecium	18	46,2	15	38,5	17	34,7	50	39,4	10	28,6	24	39,3	19	34,5	53	35,1
Lactococcus lactis subsp. *lactis*	15	38,5	8	20,5	12	24,5	35	27,6	7	20,0	29	47,5	29	52,7	65	43,0
Lactobacillus pentosus	-	-	4	10,3	8	16,3	12	9,4	2	5,7	4	6,6	4	7,3	10	6,6
Lactobacillus brevis	-	-	4	10,3	-	-	4	3,1	-	-	-	-	-	-	-	-
Lactobacillus plantarum	-	-	1	2,6	1	2,0	2	1,6	-	-	-	-	-	-	-	-
Enterococcus casseliflavus	-	-	-	-	-	-	-	-	11	31,4	2	3,3	1	1,8	14	9,3
Enterococcus faecalis	-	-	-	-	-	-	-	-	-	-	1	1,6	1	1,8	2	1,3
Enterococcus italicus	-	-	-	-	-	-	-	-	1	2,9	-	-	-	-	1	0,7
Enteroccus durans	-	-	-	-	-	-	-	-	2	5,7	-	-	-	-	2	1,3
Staphylococcus capitis	-	-	1	2,6	1	2,0	2	1,6	-	-	-	-	-	-	-	-
Streptococcus parauberis	1	2,6	-	-	-	-	1	0,8	-	-	-	-	-	-	-	-
Staphylococcus epidermidis	-		-	-	-	-	-	-	-	-	1	1,6	-	-	1	0,7
Macrococcus caseolyticus	1	2,6	1	2,6	-	-	2	1,6	-	-	-	-	-	-	-	-
Lactococcus garvieae	-	-	-	-	1	2,0	1	0,8	2	5,7	-	-	1	1,8	3	2,0
Leuconostoc citreum	1	2,6	-	-	-	-	1	0,8	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides	2	5,1	4	10,3	6	12,2	12	9,4	-	-	-	-	-	-	-	-
Pediococcus pentosaceus	-	-	1	2,6	1	2,0	2	1,6	-	-	-	-	-	-	-	-
Weissella paramesenteroides	1	2,6	-	-	2	4,1	3	2,4	-	-	-	-	-	-	-	-
Total	39	100,0	39	100,0	49	100	127	100,0	35	100,0	61	100,0	55	100,0	151	100,0

Table 3. Molecular identification of yeasts isolates of P1 and P2. The isolates were selected randomly, grouped based on co-migration in PCR-DGGE and representatives of each group were sequenced to obtain an identification for the whole group.

Yeast isolates	**M1**		**Cu 1**		**Ch 1**		**%Total P1**		**M2**		**Cu 2**		**Ch 2**		**% Total P2**	
	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%	n.	%
Candida pararugosa	6	42,9	6	46,2	5	35,7	17	41,5	1	7,1	-	-	-	-	1	3,6
Candida zeylanoides	5	35,7	1	7,7	4	28,6	10	24,4	1	7,1	-	-	-	-	1	3,6
Candida parapsilosis	-	-	4	30,8	3	21,4	7	17,1	-	-	2	28,6	5	71,4	7	25,0
Aerobasidium pullulans	-	-	-	-	-	-	-	-	1	7,1	-	-	-	-	1	3,6
Cryptococcus diffluens	-	-	-	-	-	-	-	-	2	14,3	1	14,3	-	-	3	10,7
Cryptococcus sp.	-	-	-	-	-	-	-	-	1	7,1	-	-	-	-	1	3,6
Discophareina fagi	-	-	-	-	-	-	-	-	1	7,1	-	-	-	-	1	3,6
Rhodotorula glutinis	-	-	-	-	-	-	-	-	7	50,0	4	57,1	1	14,3	12	42,9
Debaryomyces hansenii	1	7,14	-	-	-	-	1	2,4	-	-	-	-	1	14,3	1	3,6
Trichosporon coremiiforme	2	14,3	2	15,4	2	14,3	6	14,6	-	-	-	-	-	-	-	-
Total	14	100,0	13	100,0	14	100,0	41	100,0	14	100,0	7	100,0	7	100,0	28	100,0

Table 4. Identification of the bands obtained by PCR-DGGE of bacteria of P1 and P2 based on BLAST comparison in Gene Bank.

Band[a]	Closest relative	% Identity	Source[b]
1, 15, 25	*Sus scrofa*	100%	AK239829
2	*Methylobacterium* sp.	99%	EU860986
3	*Gluconobacter thailandicus*	100%	AB436559
4, 9, 12, 20, 21, 24, 27	*Lactococcus lactis* subsp. *lactis*	100%	AB008215
5, 8, 18, 19	*Delphinella strobiligena*	98%	DQ471029
6, 22	*Escherichia coli*	100%	AJ567606
7	*Propionibacterium acnes*	97%	EF670442
10	*Saccharomyces cerevisiae*	99%	EU011664
11	*Moraxella osloensis*	98%	AM161159
13, 14	*Lactobacillus helveticus*	99%	EU273820
16	*Leuconostoc pseudomesenteroides*	97%	AB326299
17	*Kocuria rhizophila*	99%	EF204382
23	*Klebsiella* sp.	100%	FJ161951
26	*Firmicutes* bacterium	100%	EF636103

[a]Bands numbered as indicated on DGGE gels shown in Figures 1 and 2.

[b]Accession number of sequence of closest relative found with Blast search.

Table 5. Identification of the bands obtained by PCR-DGGE of yeasts of P1 and P2 based on BLAST comparison in Gene Bank

Band[a]	Closest relative	% Identity	Source[b]
1	*Aureobasidium pullulans*	99%	DQ872874
2	*Phoma herbarum*	99%	EU082106
3	*Seyrigia humbertii*	98%	AY968421
4, 5, 6, 7, 8, 15	*Saccharomyces cerevisiae*	100%	GQ227687
9	*Filobasidium elegans* or *Cryptococcus magnus* or *Cryptococcus oeirensis*[c]	99%	AF181548, FN357226, FN357225
10, 16	*Candida pararugosa*	99%	GQ139517
11, 12	*Rhizomucor miehei*	100%	AF198253
13,14	*Alternaria alternata*	99%	GQ221851

[a]Bands numbered as indicated on DGGE gels shown in Figures 3 and 4.

[b]Accession number of sequence of closest relative found with Blast search.

[c]The 26S rRNA gene region was 100% identical therefore avoiding identification.

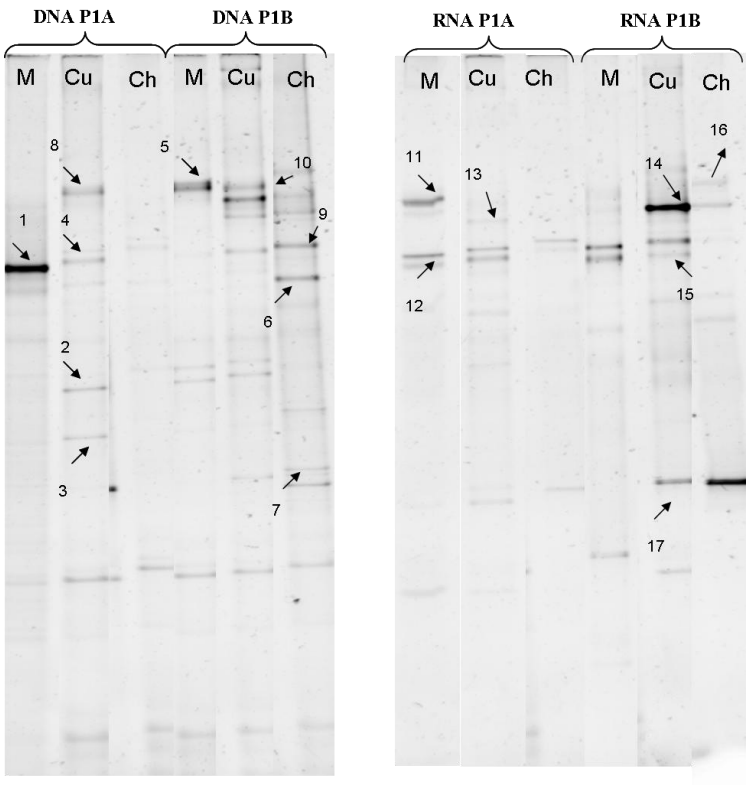

Fig. 1. Bacterial DGGE profiles of the nucleic acids extracted directly from samples of P1 and amplified with primers 338f and 518r. Bands indicated by numbers were excised and after re-amplification (as described in Materials and methods), subjected to sequencing. The identification of the bands is reported in Table 4

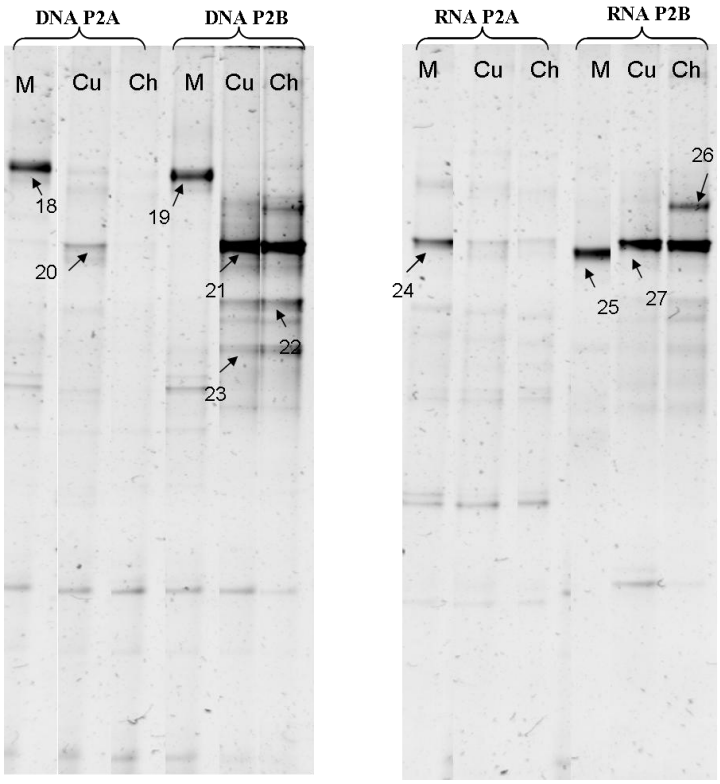

Fig. 2. Bacterial DGGE profiles of the nucleic acids extracted directly from samples of P2 and amplified with primers 338f and 518r. Bands indicated by numbers were excised and after re-amplification (as described in Materials and methods), subjected to sequencing. The identification of the bands is reported in Table 4

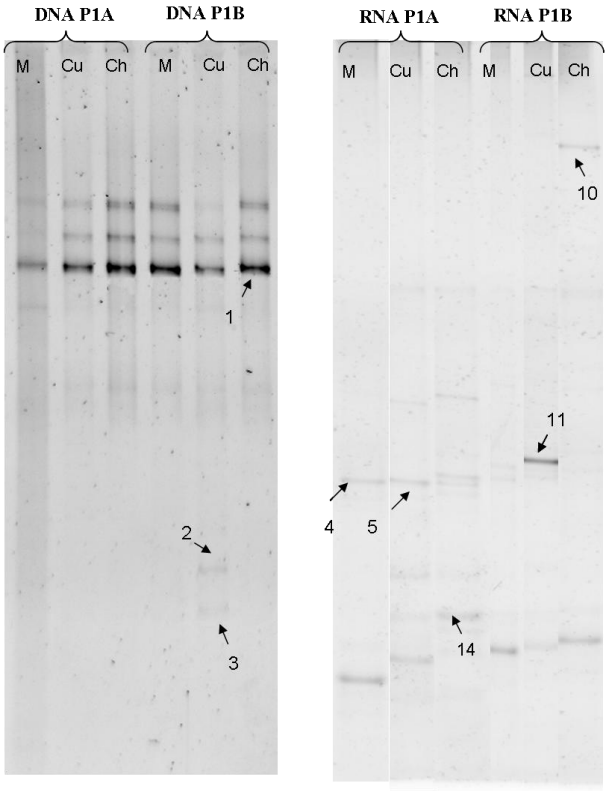

Fig. 3. Yeasts DGGE profiles of the nucleic acids extracted directly from samples of P1 amplified with primers NL1 and LS2. Bands indicated by numbers were excised and after re-amplification (as described in Materials and methods), subjected to sequencing. The identification of the bands is reported in Table 5

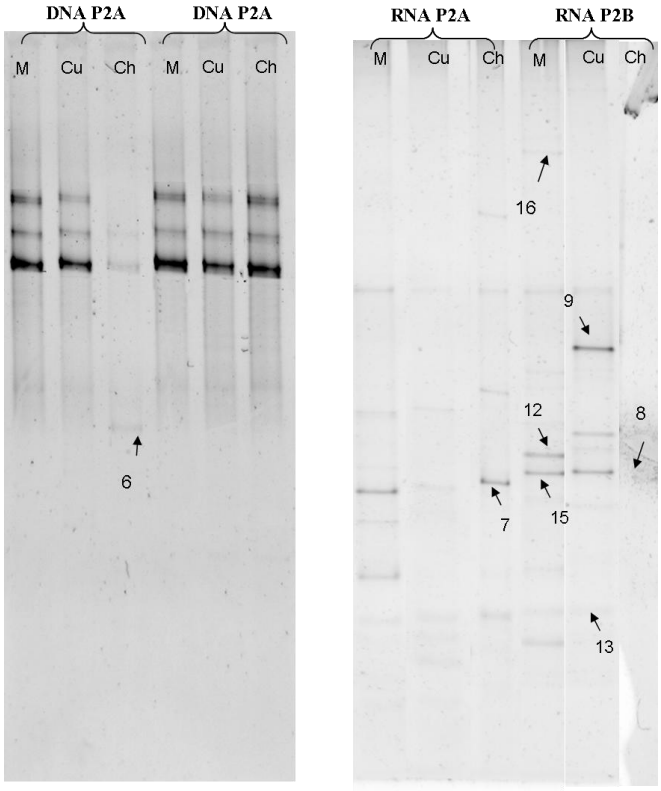

Fig. 4. Yeasts DGGE profiles of the nucleic acids extracted directly from samples of P2 amplified whit primers NL1 and LS2. Bands indicated by numbers were excised and after re-amplification (as described in Materials and methods), subjected to sequencing. The identification of the bands is reported in Table 5